P-ISSN 2586-9000
E-ISSN 2586-9027
Homepage : https://tci-thaijo.org/index.php/SciTechAsia
Science & Technology Asia
Vol. 23 No.2 April - June 2018
Page: [86 – 97]
Original research article

Acceptor Specificity of Recombinant Cyclodextrin Glycosyltransferase from *Bacillus circulans* A11

Titaporn Chaisin[1], Jarunee Kaulpiboon [1] and Natwadee Poomipark[1,*]
[1]*Faculty of Medicine, Protein Research Laboratory, Department of Pre-Clinical Science (Biochemistry), Thammasat University, Pathum thani 12120, Thailand*

Received 8 February 2018; Received in revised form 21 May 2018
Accepted 30 May 2018; Available online 30 June 2018

ABSTRACT

Cyclodextrin glycosyltransferase (CGTase) catalyzes an intermolecular transglycosylation reaction to produce functional oligosaccharides or glycosides, which can be used in several industries. In this study, the p19bBC recombinant cells containing *CGTase* gene from *Bacillus circulans* A11 were used for synthesizing CGTase. The maximum expression was obtained when the p19bBC cells were cultured at 37ºC for 24 h with 0.2 mM IPTG. The recombinant CGTase was purified up to 6-fold by 5% (w/v) starch adsorption and the specific activity of enzyme was 1.08×10^4 units/mg with a 71% yield. The 72-kDa relative molecular mass of purified enzyme was determined by 10% SDS-PAGE. In addition, the acceptor specificity of enzyme was investigated from transglycosylation reaction using β-cyclodextrin as glycosyl donor and various saccharides and flavonoids as acceptors. Among the group of saccharide acceptors, glucose gave the highest activity, followed by maltose and mannose. Within the flavonoid group, hesperidin gave the highest activity with 13.5% as compared to glucose. Due to the broad range of bioactivities of hesperidin flavonoid, and also, the possibility of new glycoside products, this study suggested that using hesperidin as an acceptor is far superior to the saccharides in terms of further applications.

Keywords: β-cyclodextrin (β-CD); Cyclodextrin glycosyltransferase (CGTase); Flavonoid glycoside; Functional oligosaccharide; Transglycosylation

*Corresponding author: natwadeep@hotmail.com
doi: 10.14456/scitechasia.2018.17

1. Introduction

Cyclodextrin glycosyltransferase (CGTase, E.C. 2.4.1.19) was firstly found in *Bacillus macerans* [1], and a large number of microorganisms have been later identified as CGTase producers [2] such as *Actinomycetes*, *Klebsiella*, *Paenibacillus*, *Thermoanaerobacterium* and *Thermoanaerobacter* species [3]. CGTase catalyzes four related reactions: cyclization (intramolecular transglycosylation), coupling, disproportionation and hydrolysis (intermolecular transglycosylation) [4] in which part of a linear oligosaccharide chain (donor) is transferred to another linear oligosaccharide (acceptor) [5]. These catalytic mechanisms of the enzyme led to interest in the synthesis of bioactive compounds such as functional oligosaccharides and glycosides [6,7]. Several reports on the transglycosylation of interesting compounds to produce useful glycosides by the action of CGTase are available. For example, the industrial production of glucosyl stevioside, a sweetener from a plant stevioside with a very bitter taste, is catalyzed by the CGTase from *Bacillus stearothermophilus* [8]. In addition, there was research into the synthesis of flavonoid glycoside. The 3^{G}-α-D-glucopyranosyl neohesperidin synthesized by transglucosylation of the CGTase from an alkalophilic *Bacillus* species, was found to be 1,500-fold more soluble in water and 10-fold less bitter than its parent neohesperidin [9]. Until now, the use of CGTase for functional oligosaccharide and glycoside syntheses has been of great interest because it can synthesize oligosaccharide and glycoside products containing more than one monosaccharide unit, whereas the transfer reaction catalyzes by the glycohydrolases can only transfer one monosaccharide unit [10].

From our previous study, a wild type CGTase, an extracellular enzyme from *Bacillus circulans* A11, was purified through immunoaffinity gel which was prepared by coupling the anti-CGTase (IgG) to CNBr-activated Sepharose 4B [11]. However, the purified wild type CGTase gave a low yield and even had an inconvenient purification. Thus, in order to increase the expression and purification yield, a recombinant CGTase gene fused with thioredoxin **(**Trx**)**, hexa-histidine **(**His6**)** and S-protein **(**S**)** at the N terminus and a proline-rich peptide **(**PRP**)** at the C terminus, was then constructed using the wild-type gene from *Bacillus circulans* A11**.** The recombinant gene was cloned using the pET-32a vector and *Escherichia coli* BL21 **(**DE3**)** as the host cell**.** The intracellular Trx-His6-CGTase-PRP fusion protein was purified to homogeneity by starch adsorption and Ni-NTA affinity chromatography with a specific activity of 2,268 units/mg protein at a 61**%** yield **[**12**].**

In this study, to decrease the purification step and increase the specific activity of enzyme, the *CGTase* gene with a signal peptide sequence from *Bacillus circulans* A11 was sub-cloned into the pET-19b expression vector and transformed into *Escherichia coli* BL2 (DE3). The expression of *CGTase* gene in *E. coli* was performed under the stronger T7/*lac* promoter with optimizing the concentration of the IPTG inducer **[**13**].** Thus, in order to synthesize the functional oligosaccharides and glycosides of bioactive compounds with monosaccharide or disaccharide, the recombinant *Bacillus circulans* A11 CGTase from the *Escherichia coli* BL21 (DE3) transformant cell harboring the p19bBC recombinant plasmid would be chosen to use as catalyst due to its better property. In addition, this study describes the acceptor specificities of the recombinant CGTase from *Bacillus circulans* A11 and the synthesis of the functional oligosaccharides and glycosides through the intermolecular transglycosylation reaction of the enzyme.

2. Materials and Methods

2.1 Bacterial strains, plasmids and chemicals

Escherichia coli BL21 (DE3) was purchased from BioLab (UK). The pET-19b expression vector was obtained from Novagen (Germany). Restriction enzymes, DNA ligase and DNA polymerase were the products of New England Biolabs Inc. (USA). Soluble potato starch, cyclodextrins, glucose, fructose, galactose, mannose, sucrose, lactose, malto-oligosaccharides and bovine serum albumin were purchased from Sigma (USA). Tapioca starch was a gift from Siam Modified Starch Co., Ltd. (Thailand). Lactulose, hesperitin, hesperidin, diosmin, rutin hydrate, quercitrin hydrate and quercetin-3-glucoside were purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Yeast extract and tryptone were obtained from Difco (USA). The commercial glucose oxidase kit was from Human GmbH (Germany). All other chemicals used were of analytical grade.

2.2 p19bBC culture

The p19bBC, a pET-19b based plasmid containing the CGTase gene with a signal peptide sequence from Bacillus circulans A11 (GenBank accession no. AF 302787) [12,14] was transformed into *E. coli* BL21 (DE3). The p19bBC transformed *E. coli* BL21 (DE3) cells were transferred from 50% glycerol stock into LB medium containing 100 µg/mL of ampicillin. The starter culture was grown overnight at 37 °C with 250-rpm rotary shaking.

2.3 Cultivation of the p19bBC recombinant cell for CGTase production

One percent (v/v) of starter inoculum was transferred into 300 mL LB medium in a 1,000 mL Erlenmeyer flask and cultured at 37 °C with 250-rpm agitation. When the turbidity of the culture at 660 nm reached 0.6, IPTG was added to the final concentration of 0.2 mM to induce CGTase expression and cultivation was continued at 37 °C for 24 h. Bacterial cells were removed by refrigerated centrifugation at 4,800 rpm for 50 min at 4 °C. Culture broth with crude CGTase was collected and kept at 4 °C for dextrinizing activity and protein determination.

2.4 Purification and characterization of recombinant CGTase

Crude CGTase from the p19bBC recombinant cell culture was purified by starch adsorption [11]. Purity of the enzyme was investigated by Native-PAGE and SDS-PAGE [15]. Determination of molecular weight of the recombinant CGTase was also performed by SDS-PAGE

2.5 Assay of CGTase activity

Dextrinizing activity

The dextrinizing activity of CGTase was assayed by measuring the decrease in absorbance of starch-iodine complex at 600 nm [16]. One unit of enzyme is defined as the amount of enzyme which produced 10% reduction in the intensity of blue color of the starch-iodine complex per minute under the described conditions.

Coupling activity

The coupling activity, a reverse reaction of the cyclization activity, was measured by the glucose oxidase method [17]. The reaction was incubated at 37 °C for 5 min and then the absorbance at 500 nm was measured. The glucose concentration is calculated from Eq. (2.1):

$$\text{Glucose conc. } (\mu\text{mol/mL}) = 5.55 \times \frac{A_{sample}}{A_{std.}} \quad (2.1)$$

The absorbance of standard solution (Astd.) was measured using 10 µL a standard glucose (5.55 µmol/mL) with 1 mL of glucose oxidase reagent. One unit of coupling activity is defined as the amount of enzyme producing 1 µmol of glucose per minute under the assay conditions.

2.6 Protein determination

Protein concentration was determined by Bradford's method [18] using bovine serum albumin (BSA) as a standard protein. An enzyme sample (100 µl) was mixed with 1 mL of Coomassie brilliant blue reagent and left for 5 min before determining the absorbance at 595 nm.

2.7 Determination of donor and acceptor specificities

2.7.1 Donor specificity

The glucose-rich donors, β-CD and tapioca starch, were investigated for transglycosylation activity by CGTase. The reaction mixture (120 µL) containing final concentration of 2% (w/v) lactulose acceptor, 1% (w/v) glycosyl β-CD or tapioca starch donors, and 670 U/mL CGTase in 20 mM phosphate buffer, pH 6.0. The reaction was incubated at 50 °C for 24 h. Then, a 5-µL aliquot was withdrawn and glucose concentration was determined by the glucose oxidase method [17]. Samples were also analyzed using thin layer chromatography (TLC) (System I). The intensity of functional glycoside or oligosaccharide product spots were quantitated relative to glucose spot in the same TLC plate. The highest intensity of functional glycoside or oligosaccharide spots were set to 100%.

2.7.2 Acceptor specificity

Purified recombinant CGTase in phosphate buffer, pH 6.0, was incubated with 1% (w/v) β-CD as a glycosyl donor and two types of acceptor: 2% (w/v) of saccharides and flavonoids. The saccharides were divided into two groups: monosaccharides and disaccharides. The group of monosaccharides were C6 saccharides such as glucose, fructose and galactose. The group of disaccharides were maltose, sucrose, lactose and lactulose. The flavonoids were hesperitin, hesperidin, diosmin, rutin hydrate, quercitrin hydrate and quercetin-3-glucoside. Each reaction mixture was incubated at 50 °C for 24 h. The products from various acceptors were analyzed by the glucose oxidase method and TLC plate using separately appropriate systems (described below).

2.8 Determination of transglycosylation reaction efficiency

Transglycosylation reaction efficiency was judged by transglycosylated product yield on TLC plate with mobile phase System I, II and III. The yield was quantitated using a GS-800™ calibrated imaging densitometer with Quantity One® 1-D analysis program (Bio-Rad Laboratories, Inc., USA). A glucose spot which was run on the same TLC plate was used as the standard.

2.9 Thin layer chromatography (TLC) analysis

2.9.1 System I for identification of the best glycosyl donor

Reaction products were analyzed on TLC silica gel 60 F_{254} (20 cm in height) (Merck, Germany), and twice developed in a System I composing of n-butanol/pyridine/water (5:4:1, v/v) as a mobile phase for finding the best donor in the synthesis of glycoside or oligosaccharide products [19]. The TLC chromatograms were visualized by dipping with concentrated sulfuric acid-methanol (1:9, v/v) followed by heating at 120 °C for 15 min. The intensity of synthesized product spots was quantitated by a scanning densitometer. Glucose spot (50 µg) was set as standard value.

2.9.2 System II for identification of the best mono- and di- saccharide acceptor

Reaction products were analyzed by applying samples on a TLC plate, and developed in a System II composed of isopropyl/ethyl acetate/water (3:1:1, v/v) as a mobile phase [20] for detection of glycosyl oligosaccharide products. After running twice, the plate was dried thoroughly and dipped in the same procedure as in system I. The intensity of glycosyl oligosaccharide product spots was evaluated from the glucose standard value.

2.9.3 System III for identification of flavonoid glycoside products

Flavonoid glycoside products were analy Flavonoid glycoside products were analyzed on a TLC plate. The mobile phase System III composed of ethyl acetate/acetic acid/water (3:1:1, v/v) was used for running a TLC plate [21]. After running once, the plate was visualized in the same procedure as in system I. The intensity of flavonoid

glycoside product spots were evaluated from the glucose standard value.

2.10 High performance liquid chromatography (HPLC) analysis

The obtained products were also analyzed by HPLC (Agilent Technologies 1260, Germany) using an Acclaim™ 3 µm C18 column (4.6 x 150 mm) (Thermo Fisher Scientific Co., LTD., Thailand) and detected with a UV detector. The reaction mixture of glycoside or oligosaccharide product was filtered through a nylon membrane of 0.45 µm disc filter before injection and eluted with acetonitrile: water (20:80, v/v) using a flow rate of 0.5 mL/min at 40˚C [9,22]. Product yield was calculated from Eq.(2.2):

$$\text{Yield } (\%) = \frac{\text{Peak area of product}}{\text{Peak area of acceptor at } t_o} \times 100 \quad (2.2)$$

3. Results and Discussion

3.1 Purification and characterization of recombinant CGTase

The expression level of crude CGTase showed a specific activity of 1.78x103 U/mg protein (Table 1). After 5% (w/v) starch adsorption, the CGTase purity was up to 6-fold with a yield of 71% and the specific activity was 1.08x104 U/mg protein (Table 1).

Table 1. Purification of recombinant CGTase from the p19bBC transformant cell

Purification step	Total protein[b] (mg)	Total activity[c] (U)	Specific activity (U/mg)	Purity fold	Yield (%)
Crude CGTase[a]	595.2	$1.1x10^6$	$1.8x10^3$	1	100
5% (w/v) Starch adsorption	69.9	$7.6x10^5$	$1.1x10^4$	6	71

[a] Crude extract was prepared from 2.4 liters (9.5 g wet weight) of cell culture
[b] Assayed by Bradford's method
[c] Assayed by dextrinizing activity

In previous reports, crude CGTase from *Paenibacillus* sp. RB01 was 46.3-fold purified with 38.0% yield after using starch adsorption [23] and that from an alkalophilic *Bacillus* species was 22.7-fold purified with 51.0% yield after using starch adsorption and subsequently, Q-Sepharose chromatography [24]. From our previous work [12] on purification of recombinant CGTase from recombinant pCD8 cell by starch adsorption and HisTrap FF^{TM} column, and pVR313-XL-1-Blue cell by starch adsorption and ultrafiltration, the yields were 61% and 39% with specific activity of 2,268 and 2,214 U/mg, respectively. These results suggest that the yield as well as the purity of enzyme depend on the expression vector and purification process. Importantly, several protocols for enzyme production with a high yield of recombinant proteins using *E. coli* host cell usually are performed under systems of overexpression vector (such as pET series) and shortest step of purification. Therefore, in our study, we ligated the A11 *CGTase* gene with pET-19b plasmid carrying an N-terminal His-Tag sequence and expressed under its T7 promoter to obtain the stable and high-level CGTase protein; also, it was convenient to purify.

3.2 Analytical gel electrophoresis

The purity of the recombinant CGTase was checked by native-PAGE and SDS-PAGE. On native-PAGE, the enzyme revealed one band on protein and dextrinizing activity staining (Fig. 1A and 1B). In addition, the activity band that showed the clear zone on the dark brown background was observed in the same position as the protein band. This result confirms that the obtained protein after a purification was the target enzyme, which obviously indicates from starch-degrading activity. When 10% SDS-PAGE was performed (Fig. 2), the enzyme showed one major band with the relative molecular weight of 72 kDa. In comparison, the molecular weight of the recombinant CGTase had the size similar to CGTase from

Paenibacillus sp. RB01 (70 kDa) [23], *B. firmus* (75 kDa) [25] and *B. macerans* IAM 1243 (74 kDa) [26], but had the different size from the CGTases from alkalophilic *Bacillus* sp. (80 kDa) [24], *B. stearothermophilus* ET1 (67 kDa) [27], and *Thermococcus sp.* B1001 (83 kDa) [28].This might be a unique characteristic of each CGTase in different species.

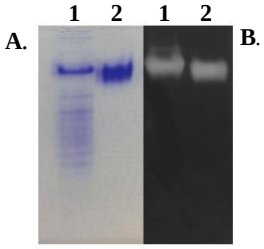

Fig. 1. Native-PAGE analysis of recombinant CGTase from the p19bBC cell (A.) Coomassie® Brilliant Blue R-250 staining (Lane 1: Crude CGTase 20 μg, Lane 2: 5% (w/v) Purified CGTase 20 μg) (B.) Activity staining (Lane 1: Crude CGTase 2 U, Lane 2: 5% (w/v) Purified CGTase 2 U)

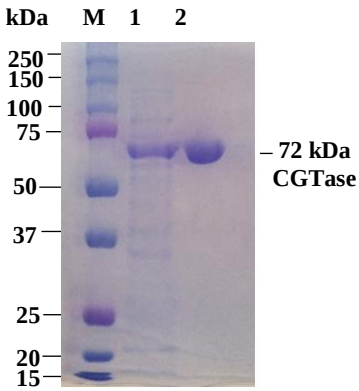

Fig. 2. SDS-PAGE analysis of recombinant CGTase in each purification step

Lane M: Molecular weight marker proteins (myosin 250 kDa, phosphorylase 150 kDa, β-galactosidase 100 kDa, bovine serum albumin 75 kDa, ovalbumin 50 kDa carbonic anhydrase 37 kDa, trypsin inhibitor 20 kDa and lysozyme 15 kDa), Lane 1: Crude CGTase 20 μg, Lane 2: 5% (w/v) Purified CGTase 20 μg

3. 3 Determination of donor and acceptor specificities

In determining donor specificity, the recombinant CGTase was incubated with β-CD or tapioca starch as a glycosyl donor and lactulose as an acceptor in 20 mM phosphate buffer, pH 6.0, at 50 ^{o}C for 24 h. The obtained products were then run on TLC and analyzed by a densitometer with Quantity One® 1-D analysis program (Fig. 3). Beta-CD was found to be a better donor, yielding 0.72 mg/mL of malto-oligosaccharide, than tapioca starch (0.71 mg/mL).

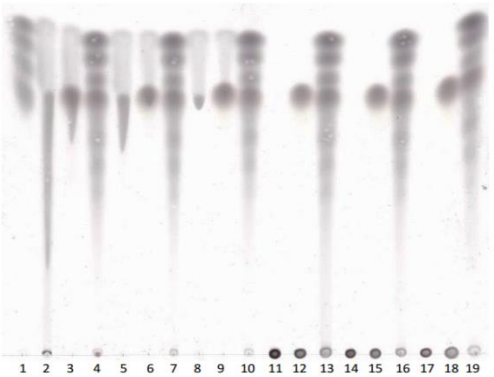

Fig. 3. TLC analysis of the products from the reaction of recombinant CGTase with β-CD or tapioca starch donor and lactulose acceptor (Lane 1: Standard G_{1-4}; Lane 2 : Standard 1% β-CD; Lane 3-4: Reaction mixture with 1% (w/v) β-CD, 0 and 24 h; Lane 5: Standard 0.5% β-CD; Lane 6-7: Reaction mixture with 0.5% (w/v) β-CD, 0 and 24 h; Lane 8: Standard 0.25% β-CD; Lane 9-10: Reaction mixture with 0.25% (w/v) β-CD, 0 and 24 h; Lane 11: Standard 1% starch; Lane 12-13: Reaction mixture with 1% (w/v) starch, 0 and 24 h; Lane 14: Standard 0.5% starch; Lane 15-16: Reaction mixture with 0.5% (w/v) starch, 0 and 24 h; Lane 17: Standard 0.25% starch; Lane 18-19: Reaction mixture with 0.25% (w/v) starch, 0 and 24 h)

The acceptor specificity of recombinant CGTase was determined from

the glucose oxidase method (Figs. 4 and 5). The recombinant CGTase in phosphate buffer, pH 6.0, was incubated with 1% (w/v) β-CD donor and two groups of acceptor: 2% (w/v) of saccharides and flavonoids. The results showed that glucose gave the highest activity which further set as 100%, followed by maltose, mannose, fructose, lactose, galactose, sucrose, and lactulose, which gave 57.3, 30.9, 29.4, 29.0, 28.8, 28.3, and 27.5% of activity, respectively, as compared to glucose. These results suggested that within the monosaccharide group, the monosaccharides with aldose functional group such as glucose and mannose are better acceptors than the ketose group.

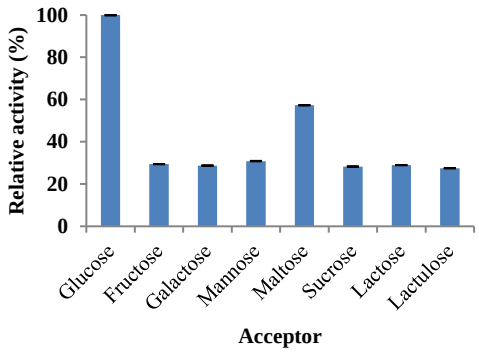

Fig 4. Saccharide acceptor specificity of recombinant CGTase. 1% (w/v) β-CD donor was incubated with 1% (w/v) mono- and di-saccharide acceptors and enzyme in 0.2 M phosphate solution, pH 6.0, at 50 °C for 24 h.

To be a good acceptor, the OH configuration of aldose at position C2 and C4 of glucose would be better than other C positions. Moreover, the C4 position of glucose seems to be more important than C2. The conclusion offered above is supported by the lower relative activity when C4 of glucose is changing into galactose (a C4 epimer of glucose) than that of C2 of glucose changing into mannose (a C2 epimer of glucose). In the disaccharide group, maltose (glucose-α-1,4-glucose) gave the highest activity, followed by lactose (galactose-α-1,4-glucose), sucrose (glucose-α-1,2-fructose) and lactulose (galactose -β-1,4-fructose). These disaccharides having at least one glucose could be better than none.

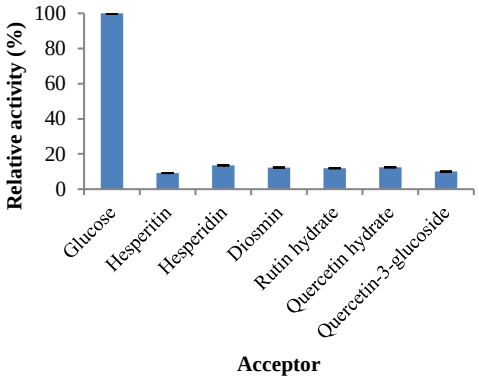

Fig. 5. Flavonoid acceptor specificity of recombinant CGTase. 1% (w/v) β-CD donor was incubated with 1% (w/v) flavonoid acceptors and enzyme in 0.2 M phosphate solution, pH 6.0, at 50 °C for 24 h.

As shown in Fig. 5, the transglycosylation to flavonoids was found to be lower than that to saccharides. This effect may be due to a solubility problem of flavonoids [24]. Comparing acceptor specificity in the flavonoid group found that hesperidin gave the highest transglysylation activity, followed by quercitrin hydrate, diosmin, rutin hydrate, quercetin-3-glucoside and hesperitin, which yielded 13.5, 12.4, 12.3, 11.9, 10.0 and 9.1% of activity, respectively, as compared to glucose. To our knowledge, only a few studies on saccharide and flavonoid acceptor specificities of recombinant CGTase have been reported. Kometani et al. [22,24] reported transglycosylation to various saccharides, and flavonoids at alkaline pH was more effective than that at neutral pH. They found that D-glucose, D-xylose, D-arabinose, L-fucose, D-fructose and D-mannose were good acceptors for CGTase from *B. stearothermophilus*, *B. circulans*, and *B. macerans*. Among flavonoids, diosmin and hesperidin were more effectively

transglycosylated by CGTase than naringin and neohesperidin. For another aspect of CGTase study, Kitahata et al. reported that *Bacillus stearothermophilus* CGTase had a wider acceptor specificity than *Bacillus macerans* CGTase. The former CGTase produced large amounts of transfer products of various acceptors such as D-galactose, D-mannose, D-fructose, D- and L-arabinose, D- and L-fucose, L-rhamnose, D-glucosamine, and lactose [29]. In contrast, these saccharide acceptors were inefficient acceptors for *B. macerans* CGTase. For the acceptor specificity of the p19bBC CGTase, we concluded that this enzyme could transfer glycosyl residue to both saccharides and flavonoid acceptors, producing oligosaccharide and flavonoid glycosides.

3.4 TLC analysis

The saccharide and flavonoid acceptors with high relative glucose oxidase activity were also analyzed on TLC plate using System I, II, and III (Fig. 6 and 7) to qualitatively detect oligosaccharide and flavonoid glycoside products.

As shown in Fig. 6 (Lane 3 and 4), when the recombinant CGTase was incubated with only β-CD donor, the enzyme could hydrolyze β-CD to linear oligosaccharide. The obtained oligosaccharide was then used as both glycosyl donor and acceptor for the synthesis of maltooligosaccharides. For other saccharide acceptors such as glucose, mannose, fructose, and galactose, at least four products were found at R_f 0.94, 0.77, 0.61, and 0.5, which could be identified to be maltooligosaccharides as compared to standard G_{1-4}.

Although several saccharides were considered as the good acceptors for this recombinant CGTase, their syntheses have been already reported. Moreover, the obtained products were not gained new finding. Thus, our more attention has been paid to flavonoid acceptor group.

Glycoside products synthesized by incubation of recombinant CGTase with β-CD donor and various flavonoid acceptors were analyzed by TLC using mobile phase System III (Fig. 7).

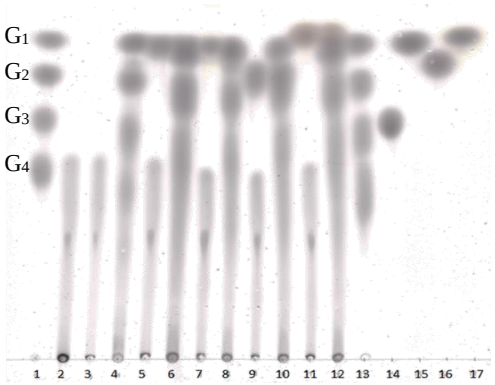

Fig. 6. TLC analysis of the oligosaccharide products from the reaction of recombinant CGTase with β-CD donor and various saccharide acceptors (Lane 1: Standard G_{1-4}; Lane 2: Standard β-CD; Lane 3-4: Reaction mixture without saccharide acceptors, 0 and 24 h; Lane 5-6: Reaction mixture with 1% (w/v) glucose, 0 and 24 h; Lane 7-8: Reaction mixture with 1% (w/v) fructose, 0 and 24 h; Lane 9-10: Reaction mixture with 1% (w/v) galactose, 0 and 24 h; Lane11-12: Reaction mixture with 1% (w/v) mannose, 0 and 24 h; Lane 13: Standard G_{1-4}; Lane 14: Standard G_3; Lane 15: Standard G_1; Lane 16: Standard galactose; Lane 17: Standard fructose)

Glycoside products synthesized by incubation of recombinant CGTase with β-CD donor and various flavonoid acceptors were analyzed by TLC using mobile phase System III (Fig. 7).

In the flavonoid group (hesperitin, hesperidin and diosmin), only the hesperidin acceptor showed glycoside products at R_f of 0.48 and 0.38, respectively. It is possible that the two products observed in the reaction, was being hesperidin glucoside and hesperidin maltoside. In the case of hesperitin and diosmin, the recombinant

CGTase could not use them as acceptors for transglycosylation.

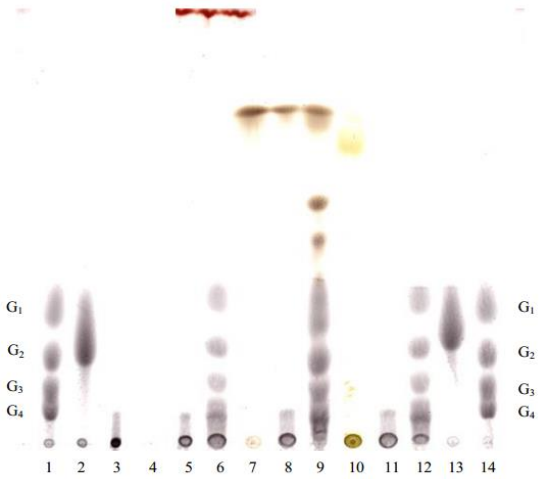

Fig. 7. TLC analysis of the glycoside products from the reaction of recombinant CGTase with β-CD donor and various flavonoid acceptors. (Lane 1: Standard G_{1-4}; Lane 2: Standard G_1; Lane 3: Standard 1% β-CD; Lane 4: Standard 2% hesperetin; Lane 5-6: Reaction mixture with 2% (w/v) hesperidin, 0 and 24 h; Lane 7: Standard 2% hesperidin; Lane 8-9: Reaction mixture with 2% (w/v) hesperidin, 0 and 24 h; Lane 10: Standard 2% diosmin; Lane11-12 : Reaction mixture with 2% (w/v) diosmin, 0 and 24 h; Lane 13: Standard G_1; Lane 14: Standard G_{1-4})

The TLC result revealed that there were only the spot of original hesperitin and diosmin, and the spot of malto-oligosaccharide products resembling the product of β-CD hydrolysis alone (Fig. 7, Lane 6 and 12). The results from this study using the recombinant CGTase were not in accordance with another report using the alkalophilic *Bacillus* sp**.** CGTase which able to use diosmin as an acceptor at alkaline pH [24]. However, the study remarkably revealed that it was unable to produce the glycoside product from diosmin at pH 5.0. Therefore, the transglycosylation is pH-dependent.

3.5 HPLC analysis

HPLC analysis was also performed to confirm a lycoside product from a hesperidin acceptor using an Acclaim™ 3 μm C18 column (4.6 x 150 mm). Considerable products were detected with a UV detector at 280 nm which is specific to flavonoids. The result revealed that at least five hesperidin glycosides (HG) were observed at R_t 8.4, 9.5, 11.5, 14.5 and 17.6 min, respectively (Fig. 8). Hesperidin recently has been found to have vitamin-like activity, which could decrease capillary permeability and fragility [30]. Its glycoside was more stable under UV radiation and strong oxidative stress than original hesperidin [24]. Therefore, these hesperidin glycosides might be suggested to apply as physiologically functional compounds for food, medicine and cosmetics with bioflavonoid (vitamin P) activity and hypotensive activity.

4. Conclusion

The p19bBC cells containing CGTase gene from *B. circulans* A11 were used for synthesizing recombinant CGTase. The crude recombinant enzyme was purified to homogeneity by a one-step starch adsorption. The specific activity of enzyme was $1.08x10^4$ units/mg with a 71% yield. This enzyme was able to catalyze transglycosylation to various saccharides and flavonoids. Among saccharides, glucose was the best acceptor for the recombinant CGTase, while in the flavonoid group, hesperidin was the most effective for the transglycosylation of the CGTase. When hesperidin was used as acceptor in the reaction catalyzed by the CGTase, at least five hesperidin glycosides (HG_{1-5}) were observed. For future industrial application, hesperidin glycoside, a functional compound, may be used as a stabilizers of pigments in cosmetic or food against UV light. Besides, HG has anti-hypertensive effects on blood pressure**.**

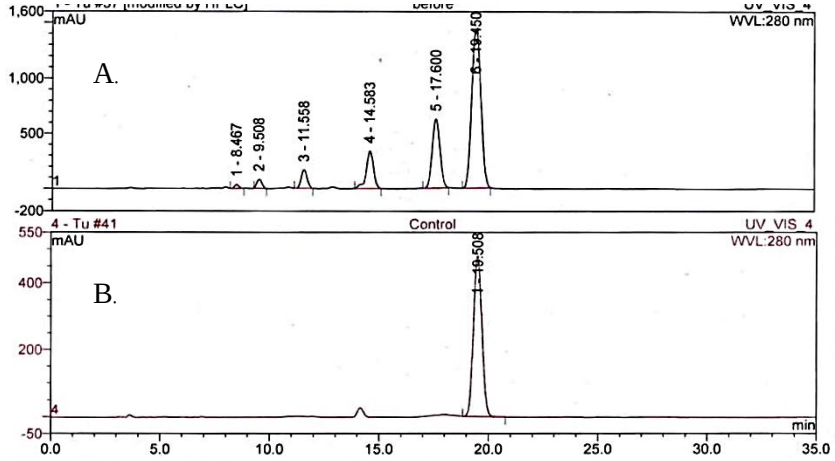

Fig. 8. HPLC chromatograms of glycoside products synthesized by the recombinant CGTase using β-CD donor and hesperidin acceptor. (A.) Glycoside products from reaction with hesperidin at 24-h incubation (B.) Control reaction without the recombinant CGTase. 1 = HG_5, 2 = HG_4, 3 = HG_3, 4 = HG_2, 5= HG_1 (Hesperidin glucoside) and 6 = H (Hesperidin)

Acknowledgements

The authors gratefully acknowledge the financial support provided by the Thammasat University Research Fund under the TU Research Scholar, Contract No. TP 2561. T. Chaisin was financially supported by a research fund from the Faculty of Medicine, Thammasat University in Year 2018. We also thank the Drug Discovery and Development Center, and Center of Scientific Equipment for Advanced Research, Thammasat University for special service rate 1 of HPLC, TLC scanner 4, and TLC visualizer.

References

[1] Tilden EB, Hudson CS. The conversion of starch to crystalline dextrins by the action of a new type of amylase separated from cultures of *Aerobacillus macerans*. J Am Chem Soc 1939;61(10):2900-2.

[2] Tachibana Y, Kuramura A, Shirasaka N, Suzuki Y, Yamamoto T, Fujiwara S, et al. Purification and characterization of an extremely thermostable cyclomaltodextrin glucanotransferase from a newly isolated hyperthermophilic archaeon, a *Thermococcus* sp. Appl Environ Microb 1999;65(5):1991-7.

[3] Zheng M, Endo T, Zimmermann W. Enzymatic synthesis and analysis of large-ring cyclodextrins. Aust J Chem 2002;55(2):39-48.

[4] Kitahata S, Okada S, Misaki A. Intermolecular transglycosylation of cyclodextrin glycosyltransferase to D-galactos. Agr Biol Chem Tokyo 1979;43(1):151-4.

[5] Biwer A, Antranikian G, Heinzle E. Enzymatic production of cyclodextrins. Appl Microb Biot 2002;59(6):609-17.

[6] Bousquet MP, Willemot RM, Monsan P, Boures E. Production, purification, and characterization of thermostable α-transglucosidase from *Talaromyces duponti* - application to α–alkyl glucoside synthesis. Enzyme Microb Tech 1998;3(1):83-90.

[7] Svasti J, Phongsak T, Sarnthima R. Transglucosylation of tertiary alcohols using cassava β-glucosidase. Biochem Bioph Res Co 2003;305(3):470-5.

[8] Malsagov M, Tugan T, Timur S, Varuzhan HA. US Patent Application Publication No.US2007/0082102 A1. Apr. 12, 2007.

[9] Kometani T, Nishimura T, Nakae T, Takii H, Okada S. Synthesis of neohesperidin glycosides and naringin glycosides by cyclodextrin glucanotransferase from an alkalophilic *Bacillus* species. Biosci Biotech Biochem 1996;60(4):645-9.

[10] Matsumura S, Imai K, Yoshikawa S, Kawada K, Uchibor T. Surface activities, biodegradability and antimicrobial properties of n-alkyl glucosides, mannosides and galactosides. J Am Oil Chem Soc 1990;67(12):996-1001.

[11] Rojtinnakorn J, Kim P, Laloknam S, Tongsima A, Kamolsiripichaiporn S, Limpaseni T, et al. Immunoaffinity purification and characterization of cyclodextrin glycosyltransferase from *Bacillus circulans* A11. Science Asia 2001;27(2):105-12.

[12] Kaulpiboon J, Prasong W, Rimphanitchayakit V, Murakami S, Aoki K, Pongsawasdi P. Expression and characterization of a fusion protein-containing cyclodextrin glycosyltransferase from *Paenibacillus* sp. A11. J Basic Microb 2010;50(5):427-35.

[13] Charoensapyanan R, Ito K, Rudeekulthamrong P, Kaulpiboon J. Enzymatic synthesis of propyl-α-glycosides and their application as emulsifying and antibacterial agents. Biotechnol Bioproc E 2016;21(3):389-401.

[14] Rimphanitchayakit V, Tonozuka T, Sakano Y. Construction of chimeric cyclodextrin glucanotransferases from *Bacillus circulans* A11 and *Paenibacillus macerans* IAM1243 and analysis of their product specificity. Carbohydr Res 2005;340(14):2279-89.

[15] Weber K, Osborn M. Proteins and SDS: Molecular weight determination on polyacrylamide gels and related procedures. In: Neurath H, Hill RL, Border C, editors. The Proteins. 2nd ed. New York, USA: Academic Press; 1975. p. 179-233.

[16] Fuwa H. A new method for microdetermination of amylase activity by the use of amylose as the substrate. J Biochem 1954;41(5):583-603.

[17] Miwa I, Okuda J, Maeda K, Okuda G. Mutarotase effect on colorimetric determination of blood glucose with β-D-glucose oxidase. Clin Chim Acta 1972;37:538-40.

[18] Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976;7(1):248-54.

[19] Saehu S, Srisimarat W, Prousoontorn MH, Pongsawasdi P. Transglucosylation reaction of amylomaltase for the synthesis of anticariogenic oligosaccharides. J Mol Catal B-Enzym 2013;88:77-83.

[20] Yoon JW, Jeon EJ, Jung IH, Min MJ, Lee HY, Kim MJ, et al. Maltosyl-erythritol, a major transglycosylation product of erythritol by *Bacillus stearothermophilus* Maltogenic amylase. Biosci Biotech Bioch 2003;67(3):525-31.

[21] Charoensapyanan R, Takahashi Y, Murakami S, Ito K, Rudeekulthamrong P, Kaulpiboon J. Synthesis, structural characterization, and biological properties of pentyl- and isopentyl-α-D-glucosides. Appl Biochem Micro 2017;53(4):410-20.

[22] Kometani T, Yoshinobu T, Nishimura T, Takashi N, Hiroshi T, Shigetaka O. Acceptor specificity of cyclodextrin glucanotransferase from an alkalophilic *Bacillus* species and synthesis of glucosyl rhamnose. Biosci Biotech Bioch 1996;60(7):1176-8.

[23] Chotipanang K, Bhunthumnavin W, Prousoontorn M. Synthesis of alkyl glycosides from cyclodextrin using cyclodextrin glycosyltransferase from *Paenibacillus* sp. RB01; 2011. p. 359-68.

[24] Kometani T, Terada Y, Nishimura T, Takii H, Okada S. Purification and characterization of cyclodextrin glucanotransferase from an alkalophilic *Bacillus* species and transglycosylation at alkaline pHs. Biosci Biotech Bioch 1994;58(3):517-20.

[25] Yim DG, Sato HH, Park YH, Park YK. Production of cyclodextrin from starch by cyclodextrin glycosyltransferase from *Bacillus firmus* and characterization of purified enzyme. J Ind Microb Biotech 1997;18(6):402-5.

[26] Takano T, Fukuda M, Monma M, Kobayashi S, Kainuma K, Yamane K. Molecular cloning, DNA nucleotide sequencing, and expression in *Bacillus subtilis* cells of the *Bacillus macerans* cyclodextrin glucanotransferase gene. J Bacteriol 1986;166(3):1118-22.

[27] Chung HJ, Yoon SH, Lee MJ, Kim MJ, Kweon KS, Lee IW, et al. Characterization of thermostable cyclodextrin glucanotransferase isolated from *Bacillus stearothermophilus* ET1. J Agric Food Chem 1998;46(3):952-9.

[28] Tachibana Y, Kuramura A, Shirasaka N, Suzuki Y, Yamamoto T, Fujiwara S, et al. Purification and characterization of an extremely thermostable cyclomaltodextrin glucanotransferase from a newly isolated hyperthermophilic archaeon, a *Thermococcus* sp. Appl Environ Microb 1999;65(5):1991-7.

[29] Kitahata S, Hara K, Fujita K, Nakano H, Kuwahara N, Koizumi K. Acceptor specificity of cyclodextrin glycosyltransferase from *Bacillus stearothermophilus* and synthesis of α-D-glucosyl O-*β*-D-galactosyl-(1→4)-β-D-glucoside. Biosci Biotech Bioch 1992;56(9):1386-91.

[30] Middleton E, Kandaswami C**.** The flavonoids**:** Advances in research since 1986**.** In**:** Harborne JB, editor**.** London**:** Chapman and Hall; 1993**.** p**.** 619**-**52**.**